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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup> :</b> <b>A61K 31/195, C12N 15/09, 15/12, C12Q 1/68, C12N 5/10, G01N 33/569</b>		<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 99/00123</b>
			<b>(43) International Publication Date:</b> 7 January 1999 (07.01.99)
<b>(21) International Application Number:</b> PCT/SE98/01232 <b>(22) International Filing Date:</b> 24 June 1998 (24.06.98)  <b>(30) Priority Data:</b> 9702457-4                      26 June 1997 (26.06.97)                      SE  <b>(71) Applicant (for all designated States except US):</b> PHARMACIA & UPJOHN AB [SE/SE]; S-112 87 Stockholm (SE).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> LIND, Peter [SE/SE]; Ringgatan 47 C, S-752 27 Uppsala (SE). WALUM, Erik [SE/SE]; Lavettvägen 21, S-184 61 Åkersberga (SE).  <b>(74) Agents:</b> TANNERFELDT, Agneta et al.; Pharmacia & Upjohn AB, Patent Dept., S-112 87 Stockholm (SE).			<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> USE OF A DRUG CAPABLE OF MODULATING THE REGULATION OF UPC-2 AND METHOD FOR SCREENING FOR POTENTIAL DRUGS AGAINST OBESITY			
<b>(57) Abstract</b>  The invention relates to a method for treatment of obesity, metabolic syndrome and/or non-insulin dependent diabetes mellitus by administering a drug capable of modulating the regulation of UCP-2, the use of a drug capable of modulating the regulation of UCP-2 for the production of drug for treatment of obesity, metabolic syndrome and/or non-insulin dependent diabetes mellitus and pharmaceutical composition comprising a pharmaceutically effective amount of such a drug. The invention is also related to methods for screening for potential drugs against obesity, metabolic syndrome and/or non-insulin dependent diabetes mellitus and the use of cDNA probe for determination of upregulation of UCP-2 for potential drugs against obesity, metabolic syndrome and/or non-insulin dependent diabetes mellitus.			

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USE OF A DRUG CAPABLE OF MODULATING THE REGULATION OF UPC-2  
AND METHOD FOR SCREENING FOR POTENTIAL DRUGS AGAINST OBESITY.

5

### Introduction

The present invention relates to method for treatment of obesity, metabolic syndrome and/or non-insulin dependent diabetes mellitus by administering a drug capable of  
10 modulating the regulation of UCP-2 and the use of a drug capable of modulating the regulation of UCP-2 for the production of drug for treatment of obesity, metabolic syndrome and/or non-insulin dependent diabetes mellitus.

It also relates to method for screening for potential drugs against obesity, metabolic syndrome and/or non-insulin dependent diabetes mellitus comprising the measurement of  
15 UCP-2 activity by biochemical, chemical or physical methods.

### Background.

Obesity is a disease with strongly increasing prevalence, and has reached epidemic proportions in the industrialized world. This disease is essentially characterized by an  
20 unbalance between energy intake and expenditure, which, without interference, leads to an ever increase in adipose tissue mass and body weight.

Appetite and energy intake is influenced by several hormonal effectors and neurotransmitters acting in the peripheral as well as the central nervous system. Examples  
25 of neurotransmitters acting to increase appetite and concomitantly body weight, are neuropeptide Y, melanin concentrating hormone, and galanin, as well as glucocorticoid hormones. Examples of hormones or neurotransmitters that counteract feeding and stimulate reduction in adipose mass are melanocortin, corticotropin releasing factor, as well as the recently described peptide hormone leptin.

Brown adipose tissue (BAT) is a well characterized tissue which is well developed in newborn mammals, including man. One important task of BAT is to generate heat and maintain body temperature homeostasis in newborns, as well as in small animals, e.g. rodents.

The uncoupling protein, UCP-1, occurs in mitochondria, and seems to be the most important protein for generating heat in BAT. It does so by burning calories using a pathway that allows dissipation of the proton electrochemical gradient across the inner mitochondrial membrane in BAT during fuel oxidation. The fuel oxidation process is uncoupled for oxidative phosphorylation of ADP to ATP, thus generating heat which is distributed from BAT to the rest of the body via the circulation. The physiological external stimulus for uncoupling activity in BAT is cold temperature. This will increase the sympathetic nervous system activity and release of catecholamines leading to stimulation of beta3 adrenoreceptors present on the surface of brown adipocytes.

Recently, a new protein denoted UCP-2 has been discovered, which is expressed not only in BAT, but also in white adipose tissue (WAT), skeletal muscle, lung, heart, placenta, etc. (Fleury C, et al. (1997) "Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia" Nat Genet 15(3), 269-272; Gimeno, RE., et al., (1997) "Cloning and characterization of an uncoupling protein homolog: a potential molecular mediator of human thermogenesis" Diabetes 46(5), 900-906). The UCP-2 protein has a 59% identity to UCP-1, and is upregulated in WAT in mice in response to feeding. This is in contrast to UCP-1, which is physiologically upregulated by cold in mice.

WO 9616031, The Upjohn Company, discloses aminoguanidine carboxylates, e.g. [1-(hydrazinoiminomethyl)hydrazino]acetic acid for the treatment of non-insulin dependent diabetes mellitus. The novel and claimed compounds reduce the abnormally elevated blood glucose level and have an increased glucose tolerance.

The invention

We have now found that a drug-induced upregulation of UCP-2 mRNA is possible.

Furthermore, we have found that, as a consequence of this, the level of UCP-2 protein  
5 increases and mitochondrial activity and heat flow increase. This serves as a foundation  
of the invention related to the drug-induced increase of metabolic efficiency, increase in  
energy expenditure, and increase thermogenesis by genetic or transcriptional upregulation  
of UCP-2 in adipose tissue. Drugs that increase energy expenditure are useful in the  
treatment of obesity, non-insulin dependent diabetes, as well as the metabolic syndrome.  
10 Obesity can be caused by different reasons such as non-insulin dependent obesity,  
increase in triglycerides, increase in carbohydrate bound energy and low energy  
expenditure.

An increase in energy expenditure includes the elevated utilization of both circulating and  
intracellular glucose and triglycerides, free or stored as glycogen or lipids as fuel for  
15 energy and/or heat production.

Our invention relates to a method for treatment of obesity, metabolic syndrome and/or  
non-insulin dependent diabetes mellitus by administering a drug capable of modulating  
the regulation of UCP-2 mRNA, thus an increase of metabolic efficiency, increase in  
energy expenditure, and increase thermogenesis by genetic or transcriptional upregulation  
20 of UCP-2 in adipose tissue. It also relates to the use of a drug capable of modulating the  
regulation of UCP-2 mRNA for the production of drug for treatment of obesity, metabolic  
syndrome and/or non-insulin dependent diabetes mellitus and pharmaceutical  
composition comprising a pharmaceutically effective amount of such a drug.

The invention is also related to methods for screening for potential drugs against obesity,  
25 metabolic syndrome and/or non-insulin dependent diabetes mellitus as defined in the  
claims and other aspects, also defined in the claims.

The measurement of UCP-2 activity as upregulation of UCP-2 transcription /mRNA can  
be done by biochemical, chemical or physical methods, all well known for persons skilled  
in the art.

Screening with pharmacological or biochemical methods can e.g. be performed on mice by the use of candidate drugs or on cell-lines such as 3T3-L1 or 3T3-F442A, that optionally can be differentiated to adipocyte like cells.

The gene for UCP-2 and a reporter-gene (e.g. E coli  $\beta$ -galactosidase, chloramphenicol acetyltransferase, alkaline phosphatase or firefly luciferase) can also be used for the measurement of the UCP-2 activity.

The invention is illustrated with four examples, using an aminoguanidine carboxylic acid, [1-(hydrazinoiminomethyl)hydrazino]acetic acid, (AG) as the substance capable of modulating the regulation of UCP-2. This is, however, no limitation of the invention in its broadest aspects.

#### Figures

- Figure 1. Upper panel: RNA blotting filter hybridized to  $^{32}\text{P}$ -labelled human uncoupling protein-2 cDNA probe.  
Bottom panel: RNA blotting filter hybridized to  $^{32}\text{P}$ -labelled human  $\beta$ -actin cDNA probe.
- Figure 2. Regulation of UCP-2 mRNA levels in white adipose and skeletal muscle tissue by AG.
- Figure 3. Effects of AG on mitochondrial activity in neuroblastoma cells.
- Figure 3. Effects of AG on reactive oxygen species in neuroblastoma cells
- Figure 5. Effects of AG on neuroblastoma cells
- Figure 6. Effects of AG on the level of UCP-protein in neuroblastoma cells

## Definitions

C5BL/6J ob/ob mice	Obese mice homozygous for a point mutation in the <i>OB</i> gene
SSPE	Sodium chloride (0.15 M), Sodium phosphate (10 mM), EDTA (1 mM), pH 7.4
SDS	Sodium dodecylsulphate
AG	[1-(hydrazinoiminomethyl)hydrazino]acetic acid

## Example 1. Upregulation of UCP-2

- 10 Six C57BL/6J ob/ob mice (Bomholtsgård, Denmark) were treated by peritoneal injection with either the compound AG (80 mg/kg) or saline (3 mice in each group). The mice were sacrificed after 20 hours and intra-abdominal fat, and skeletal muscle samples were removed from each mouse. Total RNA was extracted from these samples using the guanidinium thiocyanate method essentially as described by Sambrook *et al*, (1989)
- 15 "Molecular cloning: a laboratory manual", Cold Spring Harbor Laboratory Press, (2nd edition).

The tissues were homogenized, together with 6 ml GTC (4M guanidinium thiocyanate, 25 mM Na-citrate, 8.06 mM 2-mercaptoethanol, final adjusted pH 7.0) in a Polytron homogenizer (Brinkmann) at high speed for 1-2 minutes. 300 ml, 10% sodium lauryl

20 sarcosinate was added to a final concentration of 0.5%, then mixed thoroughly. This was centrifuged at 5000g, 10 minutes at room temperature.

The supernatant was transferred to a 50 ml Falcon tube and drawn six times through a 22-gauge needle. The tissue/GTC solution was layered on a 5.7 M cesium chloride solution (buffered by 8 mM sodium acetate and sterile filtered) and centrifuged at 32000 rpm in a

25 swing-out SW41 rotor for 17 hours at room temperature.

The pellets were dissolved in 2x200 ml diethylpyrocarbonate (DEPC) treated water and transferred to microcentrifuge tubes. One tenth of the volume of 3M sodium acetate and 2 volumes of 95% ethanol was then added, followed by precipitating the RNA in -20°C for more than 30 minutes. The RNA was collected by centrifugation at 15000g, 15 minutes

at 4°C. The pellet was washed in 70% ethanol and centrifuged for 5 minutes at 15000g. The supernatant was removed and the pellet was vacuum dried for 5 minutes. The RNA was resuspended in 100ml DEPC-treated water and kept on ice.

The integrity of the RNA was confirmed by separation on a 1% MP agarose (Boehringer Mannheim) gel. The RNA was then capillary blotted onto a GeneScreen Plus (DuPont NEN Research Products) membrane. The protocols used for the transfer and detection of the RNA are found in the technical manual: "GeneScreen and GeneScreen Plus; Hybridization Transfer Membranes; Transfer and Detection Protocols". DuPont, NEN Research Products, 549 Albany St., Boston, MA, USA.

10

The cDNA probe used for detecting UCP-2 was derived from the I.M.A.G.E. consortium clone No. 440295 and was amplified from the pT7T3D-Pac vector (Pharmacia&Upjohn) by PCR using the two primers 5'-CCAGTCACGACGTTGTAAA-3' and 5'-CACAGGAAACAGCTATGAC-3'. The probe was purified from a low-melting agarose gel prior to <sup>32</sup>P labelling which was carried out with the RediPrime DNA labelling kit kit (Amersham).

The RNA blotting filter was hybridized with the labelled probe in a 50% formamide solution at 42°C overnight with subsequent high stringency washing using four 2xSSPE for 15 minutes at room temperature, followed by two 2xSSPE, 2% SDS for 45 minutes at 65°C and, finally, two 15 minute washes in 0.1xSSPE at room temperature. After the final wash, filter bound radioactivity was detected and quantified using a PhosphorImager (Molecular Dynamics, Inc.). The results are shown in Figure 1. (top panel). After washing to remove filter-bound radioactivity, the RNA blotting filter was also hybridized to a <sup>32</sup>P-labelled probe based on human β-actin cDNA to serve as a control for the mRNA content of each lane. The results are shown in Figure 1. (bottom panel).



The relative expression levels of UCP-2 mRNA between samples of saline and AG treated mice, was calculated using the PhosphorImager radioactive counts detected within the bands corresponding to UCP-2 mRNA, and normalized against the radioactive counts within the bands corresponding to actin, the latter of which do not change significantly between saline and AG treated samples. The data (illustrated in Figure 2.) indicate a 3.6-fold induction of UCP-2 mRNA in white adipose tissue after treatment with AG, and a 1.3 fold induction in skeletal muscle.

#### Figure legends

Figure 1. Top panel: RNA blotting filter hybridized to  $^{32}\text{P}$ -labelled human uncoupling protein-2 cDNA probe. Lane 1, white adipose tissue from saline treated mice; lane 2, white adipose tissue from AG treated mice; lane 4, skeletal muscle from saline treated mice; lane 4, skeletal muscle from AG treated mice.

Bottom panel: RNA blotting filter hybridized to  $^{32}\text{P}$ -labelled human  $\beta$ -actin cDNA probe. Lane 1, white adipose tissue from saline treated mice; lane 2, white adipose tissue from AG treated mice; lane 4, skeletal muscle from saline treated mice; lane 4, skeletal muscle from AG treated mice.

Figure 2. Regulation of UCP-2 mRNA levels in white adipose and skeletal muscle tissue by AG. The white bars indicate saline treated control tissue, and the stippled bars indicate AG treated tissue. The UCP-2 mRNA levels were determined with a PhosphorImager (Molecular Dynamics) and are normalized against actin mRNA levels.

From these figures it is clearly seen that UCP-2 mRNA is strongly upregulated by AG in white adipose tissue compared to treatment with placebo (saline). This is in contrast to skeletal muscle where only a marginal change in UCP-2 mRNA levels could be seen.

Example 2. Increase in mitochondrial activity

Further experiments have shown that aminoguanidine carboxylic acid (AG) increase mitochondrial activity (Figure 3) and the generation of reactive oxygen species (ROS) (Figure 4) in human neuroblastoma cells treated for 2 days.

5

Example 3. Microcalorimetry.

In SH-SY5Y cells, as well as in L6 (rat myocytes) cells AG has been shown, by microcalorimetry, to be thermogenic. The increase in heat production initiated by the AG required several hours of incubation to establish (Figure 5).

10

Example 4. Cytometer

L6 cells and SH-SY5Y cells were shown to express UCP2 on the mRNA-level as well as the protein level. Using a laser scanner cytometer it was possible to show a shift in the peak fluorescence of antibody labelled UCP2 protein in SH-SY5Y cells after AG treatment. Increases in the protein level of UCP2 in SH-SY5Y could be detected after treatment with AG by standard flow cytometry. (Figure 6).

15

Conclusion

These data indicate the first example of a drug-induced upregulation of UCP-2 mRNA. Furthermore, we have found that, as a consequence of this, the level of UCP-2 protein increases and mitochondrial activity and heat flow increase.

20

This serves as a foundation of an invention related to the drug-induced increase of metabolic efficiency, increase in energy expenditure, and increase thermogenesis by genetic or transcriptional upregulation of UCP-2 in adipose tissue. Drugs that increase energy expenditure are useful in the treatment of obesity, non-insulin dependent diabetes, as well as the metabolic syndrome.

25

## CLAIMS

5

1. Method for treatment of obesity, metabolic syndrome and/or non-insulin dependent diabetes mellitus by administering a drug capable of modulating the regulation of UCP-2.
- 10 2. Method according to claim 1 in which the drug is capable of modulating the regulation of UCP-2 mRNA cellular levels.
3. Method according to claim 1 in which the drug is capable of modulating the transcriptional activity of the gene encoding UCP-2.
- 15 4. Use of a drug capable of modulating the regulation of UCP-2 for the production of drug for treatment of obesity, metabolic syndrome and/or non-insulin dependent diabetes mellitus.
- 20 5. A pharmaceutical composition comprising a pharmaceutically effective amount of an agent capable of modulating the regulation of UCP-2.
6. Method for screening for potential drugs against obesity, metabolic syndrome and/or non-insulin dependent diabetes mellitus comprising the measurement of UCP-2 activity  
25 by biochemical, chemical or physical methods.
7. Method according to claim 6 in which the upregulation of UCP-2 gene transcription and/or mRNA levels are measured.

8. Method for screening for an agent capable of regulating UCP-2 transcription/ mRNA comprising the steps of

- contacting an animal/or cell line with the potential agent
- 5 measuring the level of UCP-2 transcription /mRNA
- choosing the agent causing an increased level of UCP-2 transcription /mRNA in comparison to control.

9. Method for screening according to claim 8

- 10 comprising the steps of
  - contacting an animal/or cell line with the potential agent
  - measuring the difference in level of UCP-2 transcription/ mRNA between adipose and other cells
  - choosing the agent causing a higher level of transcription of UCP-2 mRNA in
  - 15 adipose than in skeletal muscle cells , indicating that the agent can modulate the regulation of UCP-2 mRNA

10. Method according to claims 8 or 9 in which the animal/or cell line is derived from adipose tissue.

20

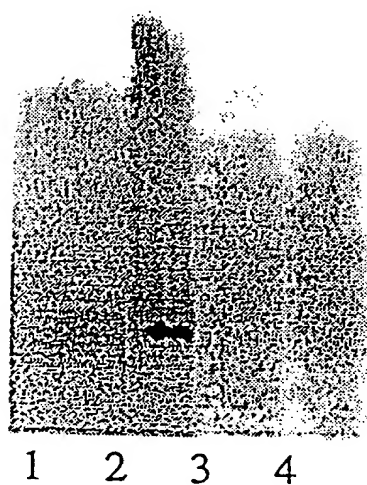
11. Use of cDNA probe for determination of upregulation of UCP-2 for potential drugs against obesity, metabolic syndrome and/or non-insulin dependent diabetes mellitus.

12. Use according to claim 11 in which adipose tissue is used.

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UCP-2 RNA blot



$\beta$ -Actin RNA blot



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Fig. 1

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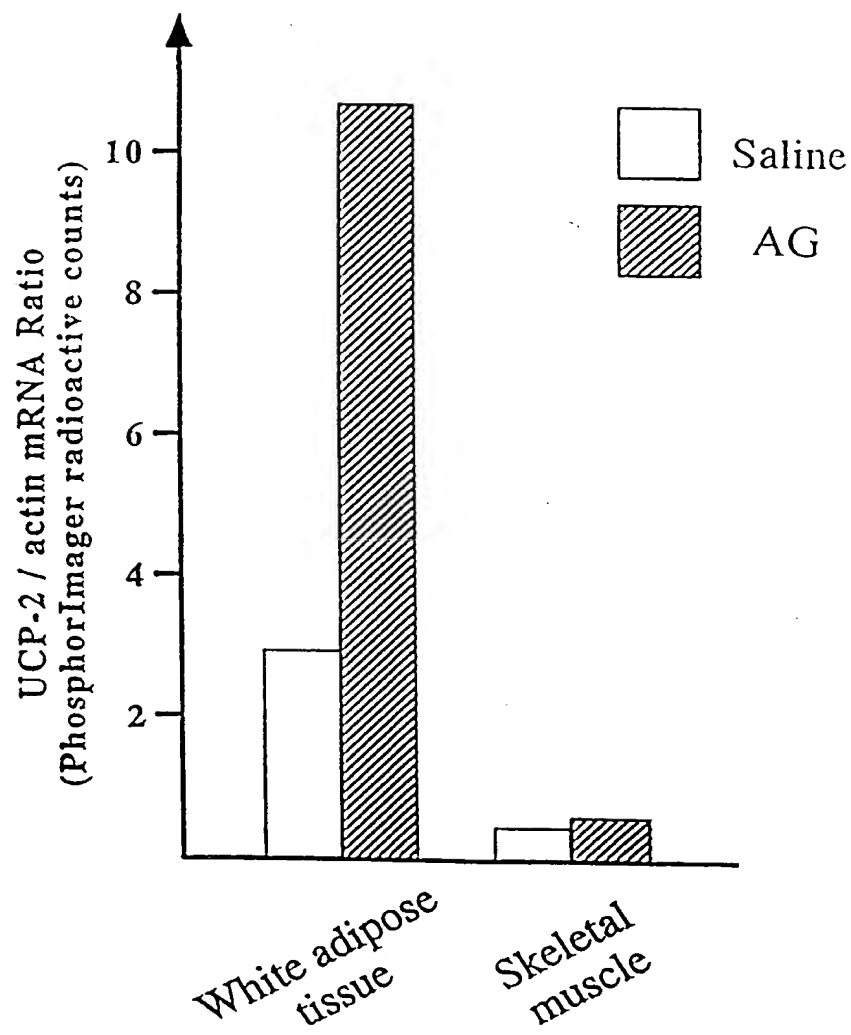


Fig. 2

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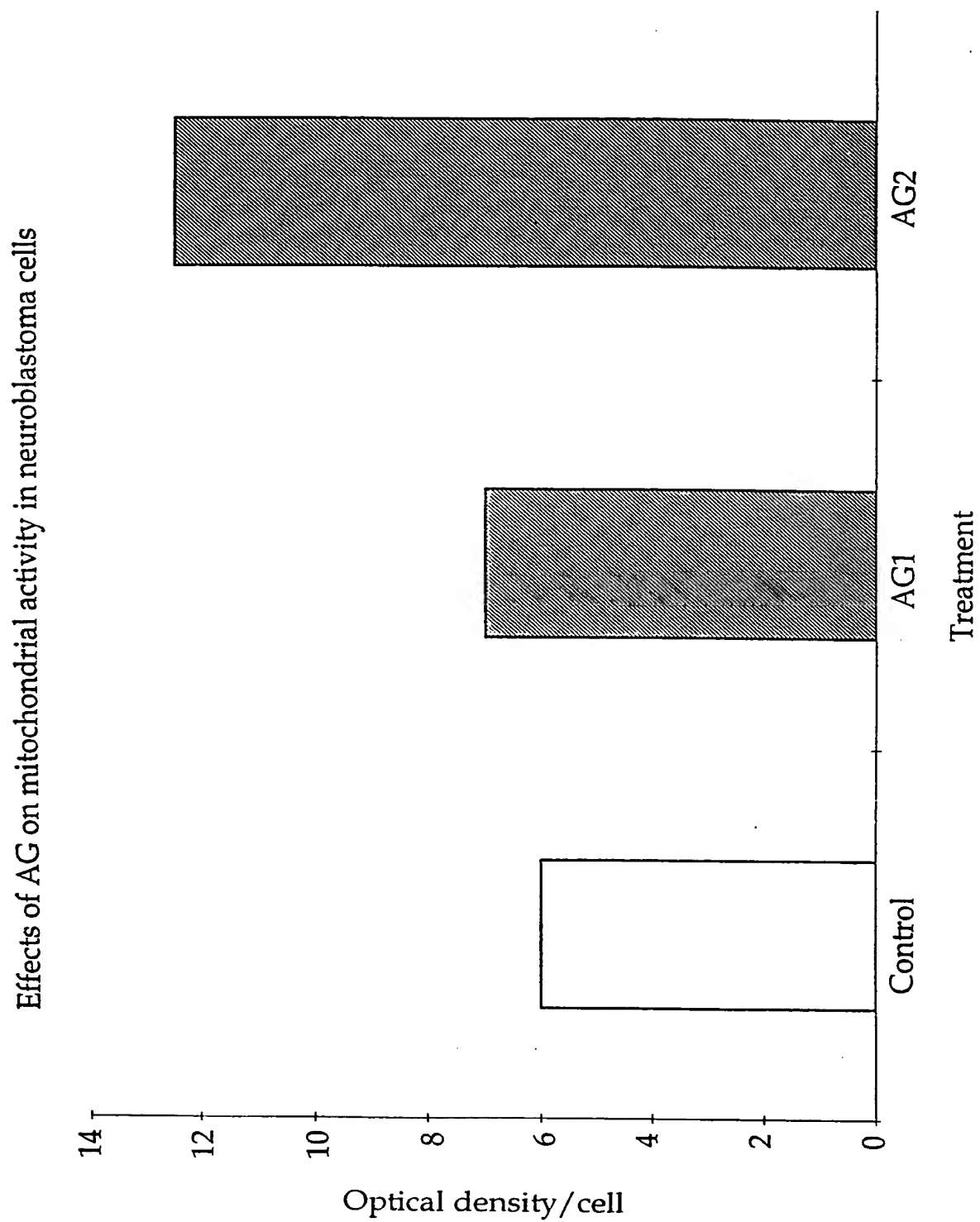


Fig. 3

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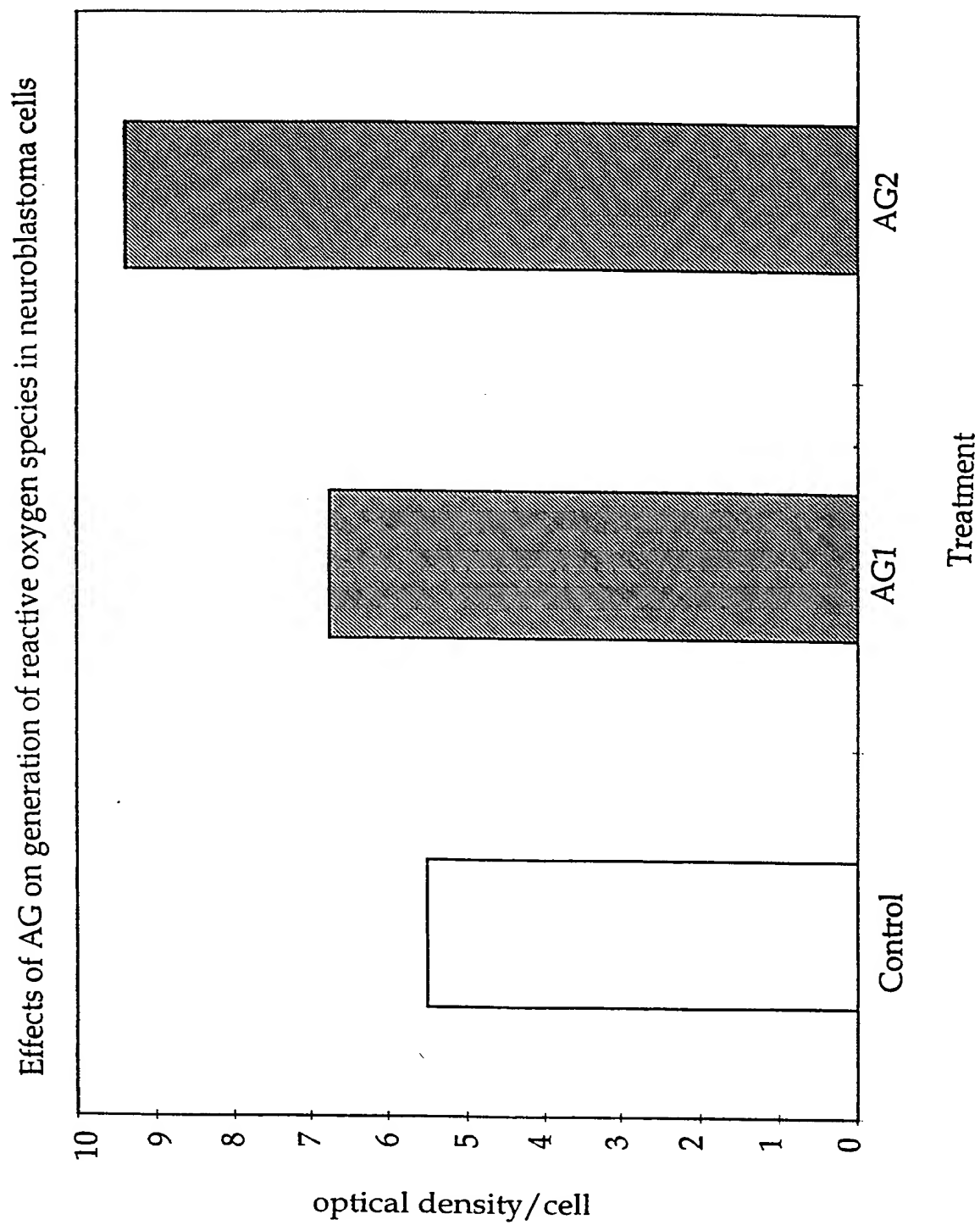


Fig. 4



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### The Effect of Aminoguanidine on Neuroblastoma Cells

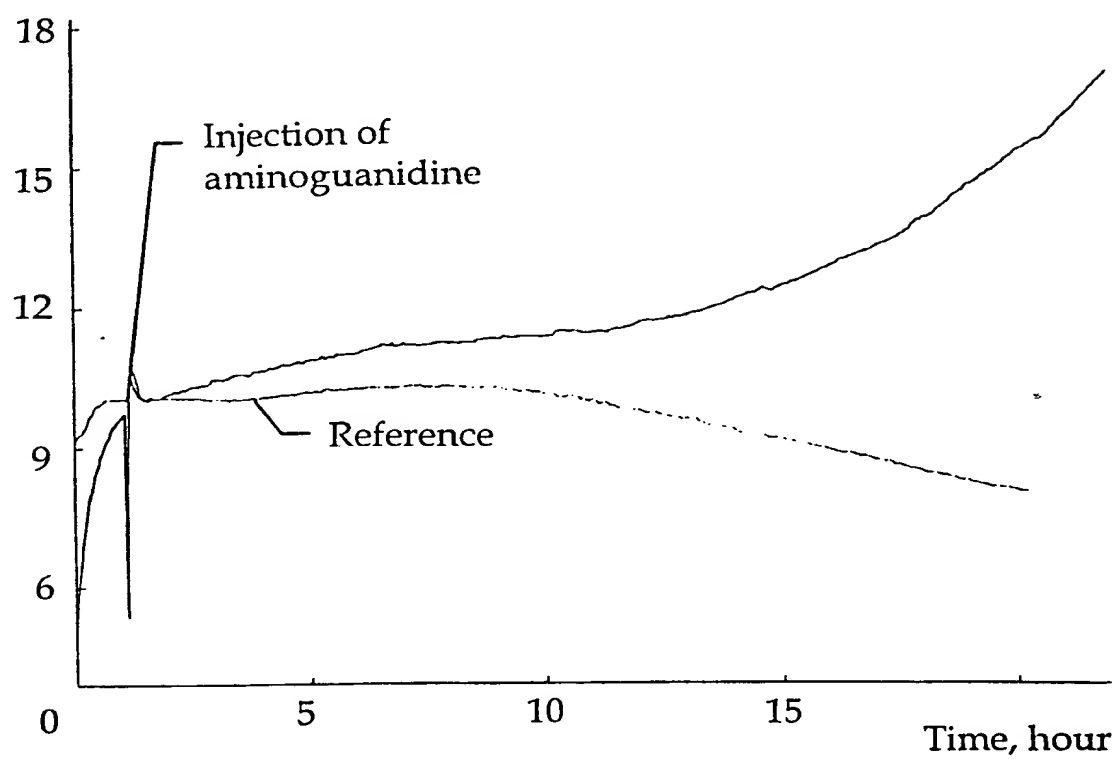
 $dQ/dt, \mu W$ 

Fig. 5

6/6

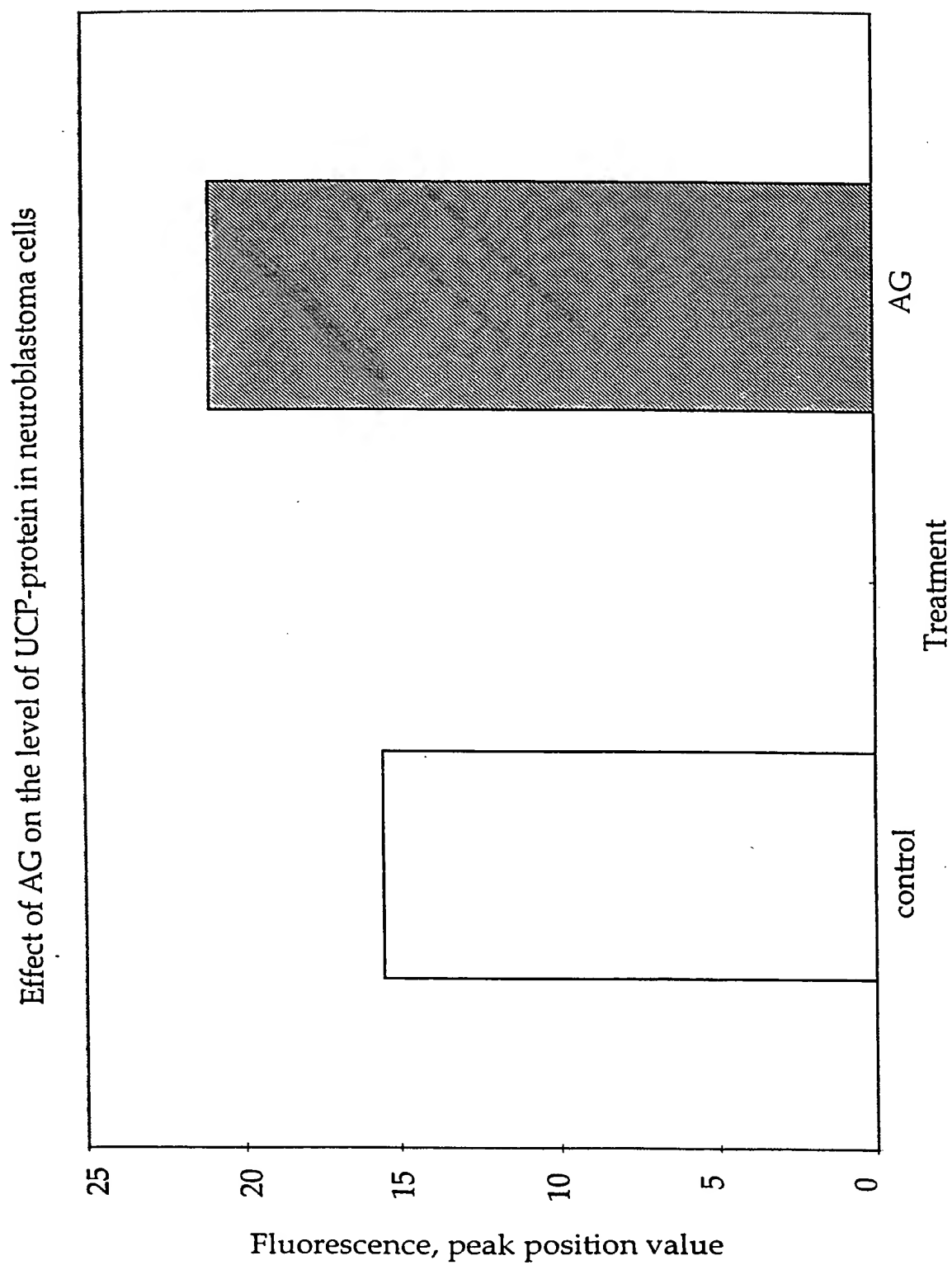


Fig. 6

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 98/01232

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 31/195, C12N 15/09, C12N 15/12, C12Q 1/68, C12N 5/10, G01N 33/569  
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9616031 A1 (THE UPJOHN COMPANY), 30 May 1996 (30.05.96) --	1-5
X	Biochemical and Biophysical Research Communications, Volume 237, 1997, Michio Shimabukuro et al, "Induction of Uncoupling Protein-2 mRNA by Troglitazone in the Pancreatic Islets of Zucker Diabetic Fatty Rats" page 359 - page 361 --	1-5



Further documents are listed in the continuation of Box C.



See patent family annex.

\* Special categories of cited documents:

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Date of the actual completion of the international search

2 November 1998

Date of mailing of the international search report

104-11-1998

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International application No.

PCT/SE 98/01232

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STN International, File CA, Chemical abstracts, volume 127, no. 16, 20 October 1997, (Columbus, Ohio, US), Okuno, Akira et al: "Effects of thiazolidines on adipose tissue of Zucker fatty rat", abstract no. 214966, Diabetes Front. (1997), 8(4), 499-501 --	1-5
A	Biochemical Pharmacology, Volume 54, 1997, Masoud Ghorbani et al, "Hypertrophy of Brown Adipocytes in Brown and White Adipose Tissues and Reversal of Diet-Induced Obesity in Rats Treated with a Beta3-Adrenoceptor Agonist" page 121 - page 131 --	1-5
A	International Journal of Obesity, Volume 21, 1997, M Ghorbani et al, "Appearance of brown adipocytes in white adipose tissue during CL 316,243-induced reversal of obesity and diabetes in Zucker fa/fa rats" page 465 - page 475 --	1-5
A	J.Clin.Invest., Volume 97, No 12, June 1996, Itsuro Nagase et al, "Expression of Uncoupling Protein in Skeletal Muscle and White Fat of Obese Mice Treated with Thermogenic Beta3-Adrenergic Agonist" page 2898 - page 2904 --	1-5
A	FR 2733513 2 (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE CNRS ETABLISS PUBLIC A CARACT SCIENT ET TECH), 31 October 1996 (31.10.96) --	1-5
P,X	WO 9831396 A1 (DUKE UNIVERSITY), 23 July 1998 (23.07.98) --	1-12
P,X	WO 9810059 A1 (TULARIK, INC.), 12 March 1998 (12.03.98) --	6-10

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 98/01232

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4623619 A (DAVID OWEBACH ET AL), 18 November 1986 (18.11.86)  --	6-12
X	WO 9640226 A1 (RONCARI, LUBA), 19 December 1996 (19.12.96)  --	1-10
X	EP 0148686 A2 (CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE), 17 July 1985 (17.07.85)  --	11-12
X	STN International, File CAPLUS, CAPLUS accession no. 1997:145811, Fleury, Christophe et al: "Uncoupling protein - 2 : a novel gene linked to obesity and hyperinsulinemia"; & Nat. Genet. (1997), 15(3), 269-272  --	1-12
P,X	STN International, File CAPLUS, CAPLUS accession no. 1998:531544, Kubota, Takanori et al: "Molecular screening of uncoupling protein 2 gene in patients with noninsulin-dependent diabetes mellitus or obesity"; & J. Clin. Endocrinol. Metab. (1998), 83(8), 2800-2804  -- -----	1-12

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 98/01232

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1-3  
because they relate to subject matter not required to be searched by this Authority, namely:  
See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

The subjects, defined by the problems and their means of the solution as listed below are so different from each other that no technical relationship can be appreciated to be present so as to form a single general inventive concept.

- 1) Claims 4-5 use of a drug capable.....and a pharmaceutical composition....
- 2) Claims 6-11 method for screening....and use of cDNA probe...

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

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